Diagnostics of fruit trees phytoplasmas – the importance of latent infections

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Abstract

In the period 2000-2008 more than 1300 fruit trees from different regions of Slovenia were tested for the quarantine phytoplasmas Apple proliferation (AP), Pear decline (PD), and European stone fruit yellows (ESFY). The majority of samples were collected within systematic official surveys, which was conducted for assessing the presence of these phytoplasma in Slovenia in production and mother plant orchards. Samples were taken from trees with and without expressed symptoms. DNA was extracted from the symptomatic shoots. In addition some roots from asymptomatic trees were sampled for the evaluation of latent infections. The presence of phytoplasmas was analyzed with a nested PCR, RFLP and a real time PCR (Hren et al., 2007). AP, PD and ESFY were confirmed as being present in several areas in Slovenia where fruit trees are cultivated. AP was found not only in apple, but also in stone fruit trees such as cherry, apricot and plum (Mehle et al., 2007). By using highly sensitive diagnostic methods, such as real time PCR, some latent infections were detected and they were confirmed next year also by less sensitive methods.

Keywords: phytoplasma, AP, PD, ESFY, latent infection, fruit trees

Introduction

Phytoplasmas are associated with many important vector borne and graft-transmissible plant diseases. They may affect different host plants including fruit trees. In Europe, the fruit trees of Rosaceae family are seriously affected by phytoplasmas belonging to the apple proliferation group (16SrX group) and causing apple proliferation (AP; 'Candidatus *Phytoplasma mali*'), European stone fruit yellows (ESFY; 'Candidatus *Phytoplasma prunorum*') and pear decline (PD; 'Candidatus *Phytoplasma pyri*') (Seemüller and Schneider, 2004).

The presence of these phytoplasmas was confirmed in several fruit-growing areas of Slovenia (Mehle et al., 2007b). Besides apple trees, AP was shown to be also present in stone fruit trees such as cherry, apricot and plum (Mehle et al., 2007a).

Symptom expression is very variable and many cultivars do not show the distinctive symptoms in the first years of tree development (Lešnik et al., 2007). In addition, it has been assumed that many trees are latently infected and disease symptoms become visible only as a result of special weather conditions or significant changes in the production practices. Such trees may be a hidden source of infection and its early detection and a consequent tree removal is as important as an intensive vector control. This is especially important in newly established orchards in areas with high disease occurrence. The main objective of our study was therefore an implementation of a real-time PCR method, which enabled the detection of the apple proliferation phytoplasma group in asymptomatic trees.

Material and methods

The sampling was done in one production and in one mother plant orchard. The majority of samples were collected within systematic official surveys, conducted to assess the presence of these phytoplasma diseases in Slovenia. Samples were taken from trees with and without expressed symptoms. DNA was isolated from the roots for analyzing latent infections or from symptomatic tree shoots using QuickPickTM Plant DNA kit (Bio-Nobile, Finland) and KingFisher mL (Thermo Scientific,USA) machine (Pirc et al., 2009; Boben et al., 2007).

The presence of phytolasmas was tested using two molecular approaches: a nested PCR followed by the RFLP analyses and a real-time PCR. Initial PCR was performed using the universal phytoplasma P1/P7 (Schneider et al., 1995) and modified primers described by Hren et al. (2007). Nested PCR reactions that followed were done using the AP group specific primers f01/r01 (Lorenz et al., 1995) and using a pair of universal phytoplasma primers U3/U5 (Lorenz et al., 1995). Products were visualized on a 1% agarose gel stained with ethidium bromide. All positive fo1/ro1 PCR products were analyzed for RFLPs (Lorenz et al., 1995) using the restriction enzymes *Ssp*I (Promega, USA) and *Bsa*AI (New England, BioLabs).

A real-time PCR procedure using the universal primers UniRNA as described by Hren et al. (2007) was employed to test the fruit tree samples for the presence of phytoplasma (Boben et al., 2007). A eukaryotic 18S rRNA TaqMan assay (Applied Biosystems, USA) was performed along with the universal testing for the presence of phytoplasmas to evaluate the efficacy of the extraction procedure. All real-time PCR reactions were run in 10 µl reaction volumes under standard conditions on 7900 HT Sequence Detection System (Applied Biosystems, USA). The results of amplifications were analyzed using SDS 2.2 software (Applied Biosystems, USA).

Results

The survey performed in one mother plant orchard revealed that especially young trees did not show typical symptoms and some infected fruit trees were symptomless (Table 1).

Tab. 1 The number of different symptomatic and asymptomatic trees, collected in one mother plant orchard (from 2004 to 2006) together with the percentage of infected trees (positive laboratory result) (Ambrožič Turk et al., 2008).

Fruit species	Symptomatic trees		Asymptomatic trees	
	No. of sampled trees	% of infected trees	No. of sampled trees	% of infected trees
Prunus persica	22	36,4	55	3,6
Prunus armeniaca	14	8,6	6	50,0
Prunus salicina	3	100,0	2	100,0
Prunus domestica	6	66,7	45	48,9
Prunus avium	3	0,0	2	0,0
Total	48	54.2	110	26.4

In autumn 2007, only one out of four real-time PCR positive samples of roots was also positive in a nested PCR analysis. Four months later, the roots of the same pear trees were sampled again. At that time, all previous real-time PCR positive samples were positive by a conventional PCR as well (Table 2).

Tab. 2 The results of an analysis of four pear trees from one plantation. Trees were sampled and analyzed twice (autumn 2007, winter 2008).

Pear	Samples (sampled 11.10.2007)	Real time PCR: phytoplasma	Nested PCR: AP group	Samples (sampled 6.2.2008)	Real time PCR: phytoplasma	Nested PCR: AP group
1	D1052/07	Pos	Neg	D53/08	Pos	Pos
2	D1054/07	Pos	Pos	D54/08	Pos	Pos
3	D1053/07	Pos	Neg	D55/08	Pos	Pos
4	D1051/07	Pos	Neg	D56/08	Neg	Neg

Discusion

The comparison of a real-time PCR with conventional PCR confirmed its higher sensitivity since phytoplasmas were also detected in some samples which were negative according to the nested PCR analysis. The sensitive real-time PCR method has been implemented in our diagnostic scheme for the detection of low concentration of phytoplasmas, which presumably appear in early infected trees and in some asymptomatic trees. However, well defined and accurate sampling of plant material is still a critical part of the detection due to uneven distribution and low concentrations of phytoplasmas in the tree.

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